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JOURNAL OF BONE AND MINERAL RESEARCH
Volume 11, Number 11, 1996
Blackwell Science, Inc.
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Purification and Characterization of Fully Functional Human Osteoclast Precursors*

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ABSTRACT

The identification and purification of human osteoclast precursors is essential to further our understanding of the mechanisms that control human osteoclast differentiation. Osteoclastoma tissue potentially provides a rich source of human osteoclast precursors, and in previous studies we have demonstrated the existence of a population of mononuclear cells within this tissue that is reactive with osteoclast-selective vitronectin receptor monoclonal antibodies. In this study, mononuclear cells expressing the vitronectin receptor, as defined by their ability to react with a murine monoclonal antibody to the β_3 chain of the vitronectin receptor (87MEM1), were isolated from collagenase digests of osteoclastoma tissue using a fluorescence activated cell sorter. Based on their fluorescence signal and size, approximately 2–3% of the viable cells (typically 2×10^5) were obtained and prepared for further phenotyping. The isolated cells demonstrated a number of phenotypic characteristics of osteoclasts: positive tartrate-resistant acid phosphatase (TRAP) activity, reactivity with human osteoclast-selective antibodies, expression of calcitonin receptors, cathepsin K (a novel osteoclast-selective cysteine proteinase) mRNA, and osteopontin mRNA and protein. These phenotypic characteristics were also detected in mononuclear cells within cryostat sections of the native osteoclastoma tissue as well as in resorption lacunae of sections of human bone. In contrast, isolated peripheral blood monocytes were negative for TRAP activity and osteopontin expression and, unlike the osteoclastoma-derived cells, demonstrated strong nonspecific esterase activity. Significantly, when the osteoclastoma-derived 87MEM1 positive cells were cocultured on whale dentine for 1–3 weeks with stromal cells, extensive resorption of the dentine surface was observed. This is the first demonstration of the purification of human osteoclast precursors. These cells provide an homogeneous cell population for studying cellular events that occur during human osteoclast differentiation. (J Bone Miner Res 1996;11:1608–1618)

INTRODUCTION

Although a number of model systems exist that have promoted the understanding of osteoclast biology, the low frequency of these cells and the difficulty in unequivocally identifying their precursors greatly restrict their study. Consequently, the identification of a homogeneous population of these cells would represent a significant advance for in vitro studies. Cell lines of osteoclast precursors would allow the study of their origins and the identification of factors that regulate osteoclastic proliferation and differentiation. Unfortunately, bone marrow (the most obvious source for osteoclasts) consists of heterogeneous populations of cells in which the specific cells of interest are present at very low frequency. Functional activity is measurable only in fully differentiated cells, and then only after

*This work was presented in part at the XII International Conference on Calcium Regulating Hormones, Melbourne, Australia, February 14–19, 1995.

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overcoming significant technical difficulties. Cell lines with osteoclastic characteristics have been reported in the literature.[1,2] Unfortunately, these cells lack the ability to resorb bone to the same degree as authentic osteoclasts, and therefore have limited utility.

In an attempt to identify a potential source of osteoclast precursors for in vitro studies, we have compared the phenotype of putative osteoclast precursor mononuclear cells in two tissue types: those detected in resorption lacunae, present in sections of human bone, and those isolated from collagenase digests of human osteoclastoma tissue. A number of observations have led to the belief that human osteoclastoma tissue could provide an ideal source of osteoclast precursors. Histologically, these rare neoplasms are characterized by the presence of mononuclear stromal cells which are irregularly interspersed with numerous multinucleated giant cells that are phenotypically indistinguishable from osteoclasts.[3] Interestingly, the unaffected bones of individuals with osteoclastomas contain normal numbers of osteoclasts. Therefore, the preponderance of these cells in the osteoclastoma tissue suggests that there are factors within this tissue that are responsible for the recruitment, proliferation, and ultimately the fusion of mononuclear osteoclast precursors to form the mature multinucleated cell. We hypothesized therefore that the precursors would be present in these neoplasms in relatively large numbers and, with the appropriate tools, could be isolated from osteoclastoma-derived cell suspensions. This is supported by our recent observation in which we demonstrated that nonadherent mononuclear cells from osteoclastomas differentiate into multinucleated osteoclasts when injected into severe combined immunodeficient mice.[4]

The osteoclast-precursor cells, present in osteoclastoma in relatively large numbers, were isolated by flow cytometry using 87MEM1, a monoclonal antibody to the β_3 chain of the vitronectin receptor.[5] Two observations prompted us to use a vitronectin receptor-reactive antibody to isolate the precursor cells: first, apart from megakaryocytes, the osteoclast and its mononuclear precursor are the only hematopoietic elements that express high levels of the vitronectin receptor[6,7]; and second, we had previously observed a population of mononuclear cells in osteoclastoma-derived cultures that bound magnetic beads coated with a vitronectin receptor-reactive monoclonal antibody.[8] Using this approach, we are able to isolate reproducibly a population of mononuclear cells from human osteoclastoma tissue that demonstrates many of the phenotypic characteristics of mono- and multinucleated osteoclasts present in resorption lacunae of cryostat sections of human bone. Significantly, using a stromal cell coculture system, these 87MEM1 positive cells isolated by flow cytometry can be induced to resorb pits in dentine slices.

MATERIALS AND METHODS

Preparation of tissue sections

A Hacker cryostat (Fairfield, NJ, U.S.A.) equipped with a tungsten-tipped steel knife (ARP, Cheshire, U.K.) was used to cut 8-μm sections of osteoclastoma and undecalcified adult human osteophytic bone.[9] These were placed on 4-well multispot slides (Henley Ltd., Loughton, Essex, U.K.), air dried, and fixed in acetone for 2 minutes. The sections were either used immediately or wrapped in foil and stored at −70°C until required.

Tartrate-resistant acid phosphatase activity

Tartrate-resistant acid phosphatase (TRAP) activity was demonstrated in the 87MEM1-positive cells and cryostat sections of osteoclastoma and undecalcified bone by the naphthol AS-BI postcoupling technique,[10] using fast red garnet GBC (BDH, Poole, U.K.) as the coupler. The preparations were incubated for 4 minutes in TRAP buffer at 37°C (0.5 mg/ml naphthol AS-BI phosphate dissolved in N,N-dimethylformamide and mixed with 0.25 M citrate buffer [pH 4.5], containing 10 mM sodium tartrate) followed by a rinse in cold distilled water. The slides were immersed in cold acetate buffer (0.1 M, pH 6.2) containing 1 mg/ml fast red garnet and incubated at 4°C for 4 minutes. The reaction was stopped by rinsing the slides in cold distilled water, and they were then mounted in 90% glycerol/10% phosphate-buffered saline (PBS) (v/v). Positive reactivity was demonstrated by the presence of a brick red/purple precipitate.

α-naphthyl acetate esterase (nonspecific esterase) activity

This was performed according to the manufacturer's protocol (Sigma Chemical Co., St. Louis, MO, U.S.A.). Prior to fixation of the sorted osteoclastoma cells and peripheral blood monocytes, 1 ml of sodium nitrite solution was added to 1 ml of Fast Blue BB solution in a glass tube. This was mixed by inversion and allowed to stand for 2 minutes before being added to 40 ml of deionized water, prewarmed to 37°C. To this mixture was added 5 ml of Trizmal buffer concentrate (pH 7.6) and 1 ml of α-naphthyl acetate solution. Following fixation in citrate-acetone-formaldehyde solution, the sections and cell populations were incubated in the substrate for 30 minutes at 37°C in the dark. The substrate was removed by copious washes in deionized water, and the cells were viewed by light microscopy. The presence of a black precipitate indicated positive reactivity.

Immunocytochemistry using C35, a human osteoclast-selective monoclonal antibody

A human osteoclast-selective murine monoclonal antibody, designated C35,[8] was used to screen cytospin preparations of the 87MEM1-positive cells (vide supra) and cryostat sections of the native osteoclastoma tissue. An alkaline phosphatase–based immunoenzymatic method was performed according to the manufacturer's instructions (LSAB kit; Dako, Carpinteria, CA, U.S.A.) to demonstrate antibody binding to the sorted cells and to cells in the native tumor tissue.

Preparation of osteoclastoma tissue for flow cytometry

Fresh osteoclastoma tissue was chopped into small pieces and placed into a sterile 50-ml centrifuge tube. The pieces were disaggregated by incubating them at 37°C for 30 minutes in serum-free RPMI-1640 medium, supplemented with 3 mg/ml (w/v) of type I collagenase (Sigma Chemical Co.). A cell suspension was obtained by gently homogenizing the remaining tissue with a plunger from a 30-ml syringe. The osteoclastoma-derived cells were resuspended in 10 ml of culture medium (RPMI with 10% fetal calf serum [FCS], 100 U/ml penicillin, and 50 μg/ml streptomycin; GIBCO BRL, Grand Island, NY, U.S.A.). These were passed through a sterile 30-μm pore Nitex membrane (Tetko Inc., Briarcliff Manor, NJ, U.S.A.) into a sterile 50-ml centrifuge tube to remove large debris. The membrane was washed with 10 ml of fresh medium, and the cell suspension was pelleted by centrifugation at 400g for 5 minutes. These cells were then used for analysis or sorting by flow cytometry.

Analysis of osteoclastoma-derived cell suspensions by flow cytometry

To determine the best source of the osteoclast precursor population, a number of osteoclastoma-derived cell suspensions were screened for the presence of antivitronectin receptor-positive mononuclear cells by flow cytometry. The cell suspensions, prepared from the osteoclastoma tissue, were resuspended in 5 ml PBS/5% FCS supplemented with 5% rabbit serum to block membrane Fc receptors. The cells were incubated on ice for 30 minutes and then aliquoted into sterile flow cytometry tubes (Falcon, Lincoln Park, NJ, U.S.A.). Following centrifugation (all centrifugation steps were performed at 400g for 5 minutes), the cell pellets were resuspended in either 87MEM1, IgG2a isotype control (Maine Biotechnology Services, Inc, Scarborough, ME, U.S.A.) or medium control (both antibodies were diluted 1:3 in RPMI/10% FCS). These were incubated on ice for 30 minutes and then washed twice, by centrifugation, in ice-cold wash buffer (PBS/5% FCS). Fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG (Sigma Chemical Co.) was added to each tube and incubated for an additional 30 minutes on ice. To remove excess conjugate, the cells were washed twice in ice-cold PBS and finally resuspended in fresh ice-cold buffer. To identify dead cells during sorting, 10 μg/ml of propidium iodide (Molecular Probes, Eugene, OR, U.S.A.) was added to each tube. The cells were then analyzed using a Becton Dickinson FACScan (San Jose, CA).

Phenotyping the osteoclastoma-derived 87MEM1 positive cells

Osteoclastoma-derived cell suspensions from a single tumor, designated GCT1293, were labeled with 87MEM1 and the controls with IgG2a, as described above. Following labeling with the FITC conjugate, aliquots of the cells were colabeled with phycoerythrin (PE)-conjugated antibodies: CD19 (B-cells), CD3 (T-cells), CD14 (monocytes), CD34 (immature stem cells), HLADR, IgG1 control, and IgG2a control. Briefly, the cell pellet in each tube was gently resuspended, and 100 μl of cold wash buffer (PBS/5% FCS) was added, followed by 40 μl of the appropriate PE-labeled antibody. The cells were mixed and incubated on ice for 30 minutes in the dark. Following two more washes, the cells were finally resuspended in cold PBS, and the appropriate volume of propidium iodide (10 μg/ml) was added.

Sorting by flow cytometry

Osteoclast precursor cells: GCT1293 cells were prepared for sorting according to the flow cytometry protocol above. FITC-labeled cells were sorted using a Becton Dickinson FACStar Plus cell sorter. These cells were either used for further phenotyping or for resorption experiments.

Peripheral blood monocytes: Human mononuclear cells were isolated from peripheral blood using a standard density gradient technique.[11] The monocytes were purified from this population using the monoclonal antibody CD14 (Dako). The localization of the primary antibody and the isolation of positive cells by flow cytometry was performed as described above. The $CD14^+$ cells were seeded onto dentine slices and cultured for 7 days at 37°C.

Histological staining of the 87MEM1 positive cells: Aliquots of the 87MEM1 positive cells were immobilized on glass microscope slides (approximately 10^3/slide) by centrifugation at 500 rpm for 1 minute, using a Shandon Cytospin 3 (Pittsburgh, PA). The cells were air dried and stained with a modified Rowmanowsky-Wright method (Diff Quik; Baxter Healthcare, McGaw Park, IL, U.S.A.) in which the slides were sequentially immersed in fast green in methanol, eosin G, and methylene blue/azure A. Finally, the slides were rinsed in tap water, air dried, and mounted in Cytoseal 280 (Stephens Scientific, Riverdale, NJ, U.S.A.). They were viewed using an Olympus AH-3 microscope.

Demonstration of calcitonin receptors

This technique was based on that described by Hata et al.[12] The 87MEM1 positive cells were resuspended in RPMI medium supplemented with 70% osteoclastoma-conditioned medium (RPMI medium/10% FCS cultured in the presence of collagenase digest preparations of osteoclastoma cells until confluent). The suspension was dispensed onto 4-well multispot slides in 0.2-ml aliquots at a density of 10^3 cells/well. After 3 days in culture, the cells were rinsed in alpha modified essential medium (α-MEM)/0.1% bovine serum albumin (BSA) and incubated with 1 μCi/ml of ^{125}I-labeled human calcitonin (2000 Ci/mmol; Amersham, Arlington Heights, IL, U.S.A.) in the presence or absence of excess unlabeled (1 μg/ml) salmon calcitonin (Peninsular Laboratories, Belmont, CA, U.S.A.) for 2 h at room temperature. The labeled calcitonin was removed by rinsing the slides three times with serum-free α-MEM/0.1% BSA. The cultures were fixed for 5 minutes in 0.2 M cacodylate buffer containing 2% gluteraldehyde (Sigma Chemical Co.). Excess fixative was removed by washing in distilled water, and TRAP activity was demonstrated in the cells as described above. The slides were dried and dipped

TABLE 1. PHENOTYPING OF THE 87MEM1-LABELED CELLS AND PERIPHERAL BLOOD MONOCYTES

	CD19	*CD3*	*HLADR*	*CD34**	*CD14**	*IgG1 control*	*IgG2a control*
$87MEM1^+$ monos	negative	negative	0.5% positive	negative	negative	negative	negative
Peripheral blood monocytes	negative	negative	positive	negative	positive	negative	negative

*There were populations of cells that demonstrated positivity with these antibodies but were distinct from the $87MEM1^+$ cells, i.e., they did not demonstrate double labeling.

in emulsion (LM-1; Amersham), diluted 1:2 in warm distilled water. After drying the slides on a cold plate, they were placed in a light-tight box and left for a few hours until completely dry. Finally, the slides were wrapped in slide trays with foil and left for 3 weeks at 4°C. The slides were developed in Kodak developer, and the extent of the signal was assessed by the autoradiographic grain density over the cell.

Demonstration of osteopontin and cathepsin K mRNA by in situ hybridization

RNA probes: pBluescript SK containing the coding region of human osteopontin was obtained from Dr. Marian Young (NIH, Bethesda, MD, U.S.A.) and prepared for use as described previously.[13] pBluescript SK^- containing a portion of the coding region of cathepsin K (also termed cathepsin O and OC2), a novel osteoclast-selective human cysteine proteinase,[14–17] was also used to generate riboprobes for in situ hybridization. The plasmid was cloned from a human osteoclast library, and the cDNA template was linearized with XhoI then transcribed from the T3 promoter to generate the sense strand (negative control). To generate the antisense (positive) strand, the template was linearized with EcoRI and then transcribed from the T7 promoter. Riboprobes were prepared using the In Vitro transcription kit method (Promega, Madison, WI, U.S.A.) with ^{35}S-thio CTP (Amersham). Following transcription, cDNA templates were digested with RQ1 RNAse-free DNAse I (Promega), and unincorporated nucleotides were removed by centrifugation through Quick Spin Sephadex G-50 columns (Boehringer-Mannheim, Indianapolis, IN, U.S.A.). In situ hybridization was performed as described by Zeller and Rogers.[18]

Induction of resorption

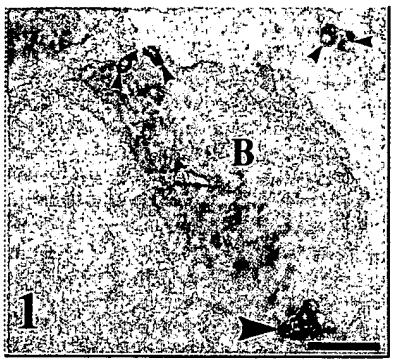
Initially, a time-course experiment was set up to determine if multinucleation and resorption could be induced in the 87MEM1-positive cells in the absence of a stromal cell population; 87MEM1-positive cells were seeded onto dentine slices (10^4/slice, in duplicate) in the presence of 70% osteoclastoma-conditioned medium (either in the presence or absence of 10^{-8} M 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$]). Duplicate slices were removed from each treatment after 7, 14, 21, and 28 days, stained for TRAP activity, and screened for resorption using a Nikon/Lasertek ILM21W laser confocal microscope, which incorporates an automatic X-Y scanning stage (Melville, NY, U.S.A.).

In a second set of experiments an attempt was made to induce the 87MEM1-positive cells to resorb by coculturing them with osteoclast-depleted stromal cells,[19] derived from osteoclastoma tissue. Osteoclast-depleted stromal cells, 1.5×10^3, were seeded onto each of two dentine slices and were cultured for approximately 4 h in wells of a 48-well plate to allow them to adhere. Approximately 2×10^5 87MEM1-positive cells were seeded onto one of the dentine slices, and these were placed at 37°C overnight. The excess buffer was removed from the slices, and these were placed into fresh wells of a 48-well plate containing 0.5 ml of 70% tumor conditioned medium in EMEM supplemented with 10^{-8} M $1,25(OH)_2D_3$. These were cultured for 1–3 weeks with a medium change once a week. After washing in PBS, the slices were analyzed for TRAP activity, as described above. TRAP-positive cells were observed by light microscopy. A selection of the slices were then sonicated and scanned for resorption pits, as described above. The remaining slices were scanned with the cells still present on their surface.

Dentine slices were prepared for scanning electron microscopy by sonicating them in distilled water for 5 minutes. Following drying in a vacuum desiccator the slices were mounted on SEM stubs, grounded with graphite, coated with Å 10–20 nm carbon in a vacuum evaporator and then examined with an Hitachi (Tokyo, Japan) S-570 scanning electron microscope operating at 15 kV.

RESULTS

Mononuclear cells expressing a number of osteoclast phenotypic characteristics were observed in cryostat sections of adult human osteophytic bone. These cells were often situated within resorption lacunae in close proximity to multinucleated osteoclasts. They demonstrated TRAP activity, reactivity with C35[8] and 87MEM1,[3] murine monoclonal antibodies selective for human osteoclasts (Fig. 1), and demonstrated osteopontin mRNA and protein.[13] These reactivities have also been demonstrated in a population of mononuclear cells in cryostat sections of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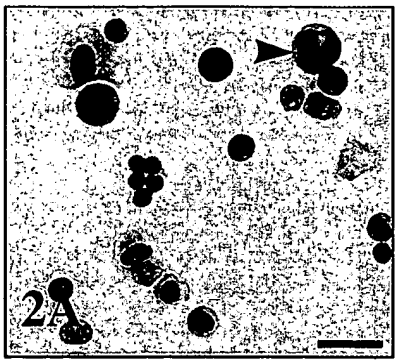
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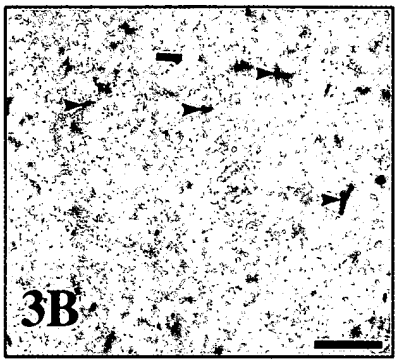
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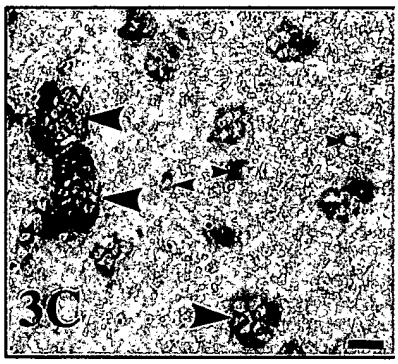
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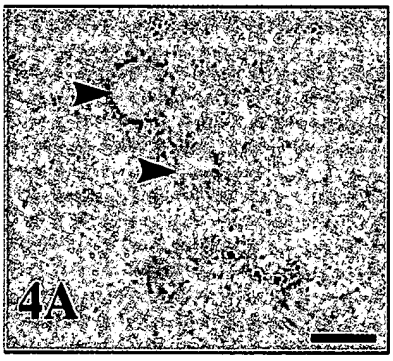
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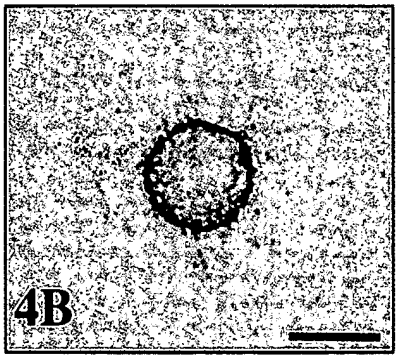
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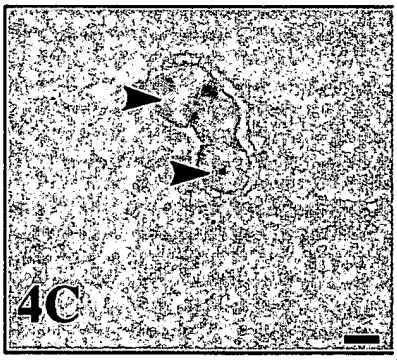
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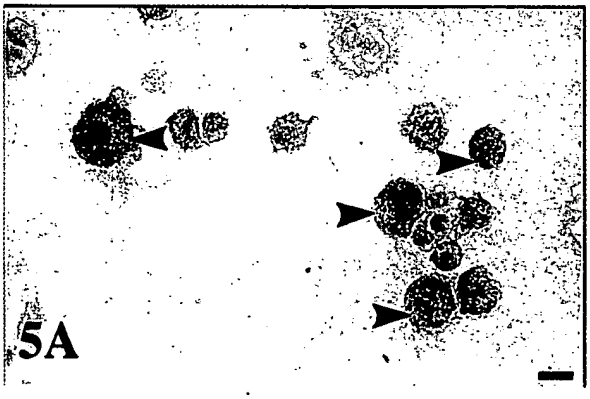
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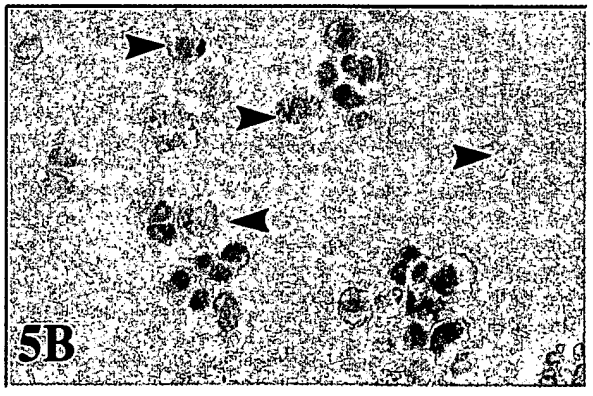
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osteoclastoma tissue.[3,8] Since these cells were present in osteoclastoma in relatively large numbers, this tissue was used as a source from which these cells could be purified.

Flow cytometry analysis of osteoclastoma-derived cells

The cells derived from five tumors were evaluated by flow cytometry to determine the best combination of viability and the percentage of 87MEM1-positive cells. Using propidium iodide, it was demonstrated that approximately 60–70% of the cells in these preparations were viable, and of these, between 0.6 and 2.5% were 87MEM1-positive mononuclear cells.

The isolated 87MEM1-positive cells did not express antigens expressed by cells of the B-cell, T-cell, and monocytic lineages (Table 1). In addition, no reactivity was detected with an antibody that recognizes an antigen on immature hematopoietic cells (CD34), and only approximately 0.5% of the 87MEM1-positive cells expressed the HLA-DR antigen.

Morphology of the 87MEM1-positive cells

The modified Rowmanowsky staining demonstrated that the 87MEM1-positive cells consisted primarily of mononuclear and occasional binucleate cells (Fig. 2A). After 7 days of culture in medium supplemented with 70% osteoclastoma-conditioned medium, multinucleated cells were observed (three or more nuclei; Fig. 2B).

Phenotyping the 87MEM1-positive cells

One of the difficulties in isolating mononuclear cells of an osteoclast phenotype is distinguishing them from monocytes or macrophages. To address this problem, we have demonstrated a number of osteoclast phenotypic markers on the 87MEM1-positive cells that provide the distinction between the two related but distinct lineages (vide infra).

TRAP and NSE activity

The 87MEM1-positive cells demonstrated TRAP activity after overnight culture on glass slides, and this activity was greatly enhanced after culturing the cells on dentine slices (Fig. 3A). These 87MEM1-positive cells did not express NSE activity. In contrast, the peripheral blood monocytes, sorted according to their CD14 reactivity, did not express any TRAP activity after culture either on glass or dentine. Also, contrary to what was observed with the 87MEM1-positive cells, these cultured monocytes did demonstrate high levels of NSE activity (Fig. 3B). In cryostat sections of the native osteoclastoma, from which the osteoclast precursor population was isolated, many TRAP-positive, NSE-negative mononuclear cells were detected (Fig. 3C).

Calcitonin receptors

The isolated 87MEM1-positive cells expressed calcitonin receptors as indicated by the localization of silver grains over these cells following autoradiography (Figs. 4A and

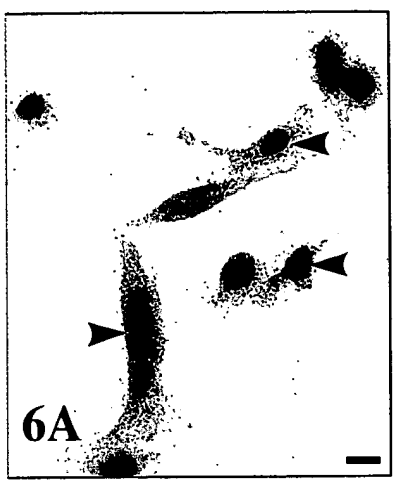
FIG. 1. (Opposite) C35 reactive mononuclear cells apposed to the bone surface in adult human osteophytic bone. Mononuclear cells reactive with C35, a human osteoclast-selective monoclonal antibody,[8] were detected in adult human osteophytic bone (small arrowheads). These cells were often apposed to the bone surface (B) and in close proximity to multinucleated osteoclasts (arrowheads). Avidin/biotin alkaline phosphatase/hematoxylin counterstain. Bar = 50 μm.

FIG. 2. (Opposite) Morphology of the osteoclastoma-derived 87MEM1-positive cells. (A) Cytospin preparations of the 87MEM1-positive cells were stained using a modified Rowmanowsky method. The majority of cells were mononucleated, but some binucleate cells were also present in the preparations (arrow). Modified Rowmanowsky. Bar = 25 μm. (B) After 7 days of culture in osteoclastoma-conditioned medium, large multinucleated cells were observed (arrowhead) among the mononuclear cells. Modified Rowmanowsky. Bar = 50 μm.

FIG. 3. (Opposite) (A) TRAP activity was demonstrated in the 87MEM1-positive cells that had been cultured on dentine (small arrowheads). In a 28-day time-course experiment, we attempted to induce resorption of the dentine by culturing the cells in the presence of osteoclastoma-conditioned medium and $1,25(OH)_2D_3$. Although no resorption was detected under these conditions, the cells remained TRAP positive. Bar = 50 μm. (B) Although no TRAP activity could be detected in isolated peripheral blood monocytes that had been cultured on dentine, they did demonstrate strong NSE activity (small arrowheads). Bar = 50 μm. (C) In cryostat sections of GCT1293, the tumor from which the osteoclast precursors were isolated, a population of mononuclear cells was observed that demonstrated strong TRAP activity (small arrowheads). TRAP-positive osteoclasts are also seen (arrowheads). Bar = 50 μm.

FIG. 4. (Opposite) Demonstration of calcitonin receptors. (A) Silver grains could be detected over the cells cultured in the presence of ^{125}I-labeled calcitonin (arrowheads). Autoradiography. Bar = 10 μm. (B) A mononuclear cell, expressing calcitonin receptors, at a higher magnification using autoradiography. Bar = 10 μm. (C) In cell preparations that were also cultured in the presence of excess cold calcitonin, no grains could be detected over the cells (arrowheads). Autoradiography. Bar = 10 μm.

FIG. 5. (Opposite) Immunolocalization of an osteoclast-selective monoclonal antibody on the 87MEM1-positive cells. (A) Strong and selective staining was detected with the human osteoclast-selective monoclonal antibody, C35, against cytospin preparations of the 87MEM1-positive cells (arrowheads). Avidin/biotin alkaline phosphatase/hematoxylin counterstain. Bar = 10 μm. (B) In contrast, no reactivity was detected with identical sorted cell cultures that had been overlayed with the negative IgG1 isotype control (arrowheads). Avidin/biotin alkaline phosphatase/hematoxylin counterstain. Bar = 10 μm.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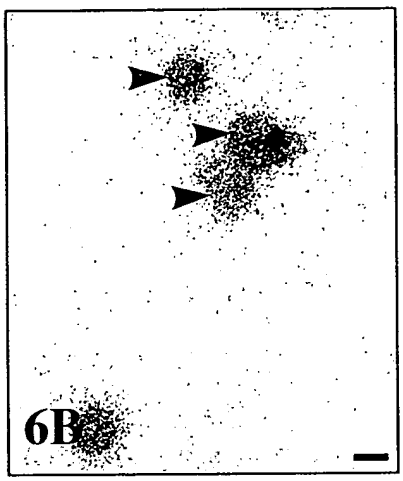
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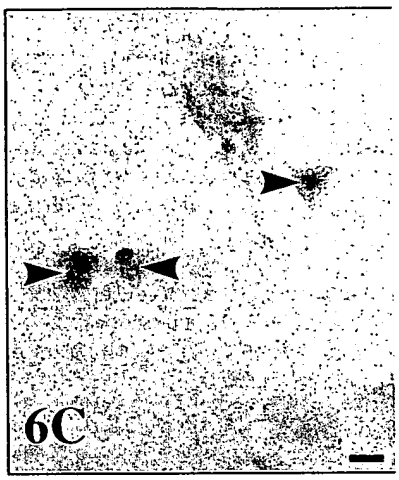
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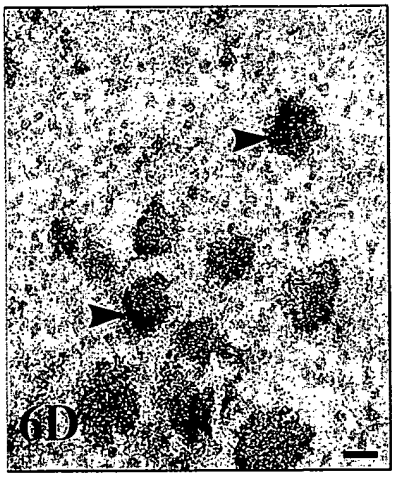
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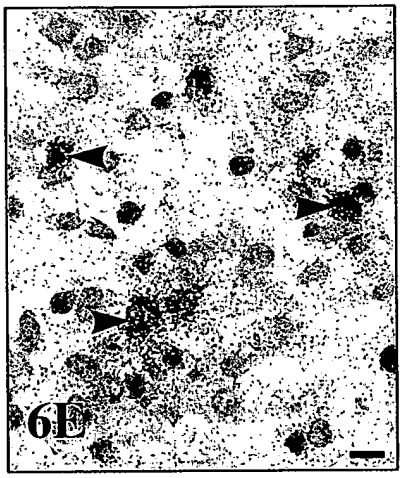
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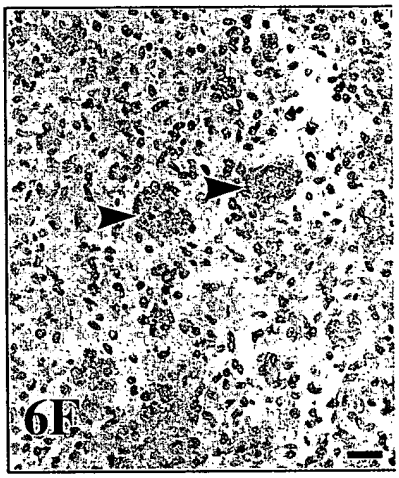
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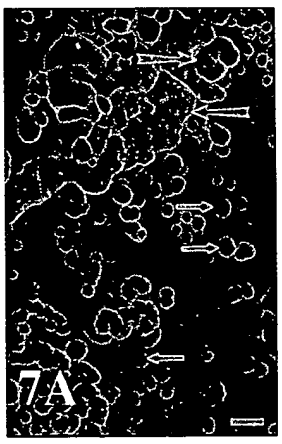
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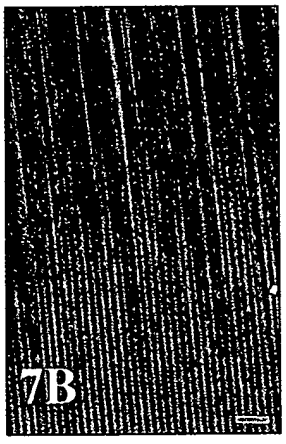
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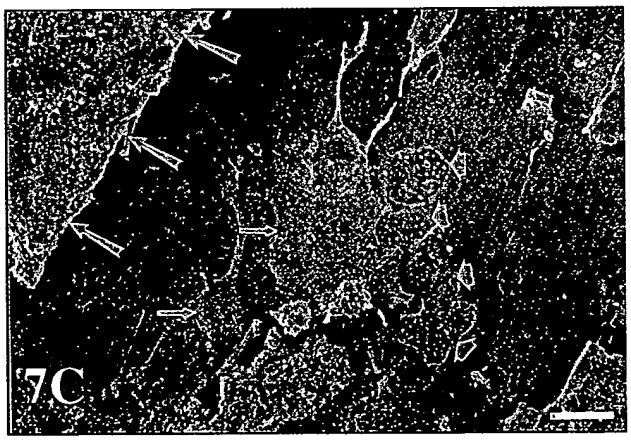
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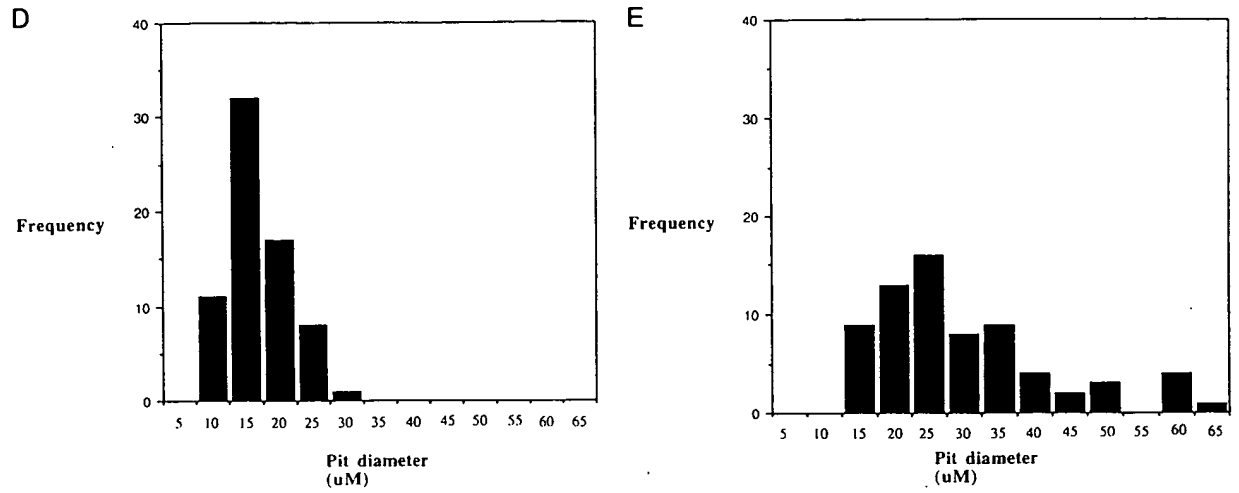

4B). The specific nature of the binding was demonstrated by the absence of grains over cells that had been cultured in the presence of unlabeled calcitonin (Fig. 4C).

C35 immunocytochemistry

Positive reactivity was detected in the 87MEM1-positive cells that had been probed with the human osteoclast-selective monoclonal antibody C35 (Figs. 5A and 5B). This reactivity was also seen in a population of mononuclear cells in cryostat sections of the native tumor (data not shown).

In situ hybridization of osteopontin and cathepsin K

Strong and specific signals were detected on preparations of the 87MEM1-positive cells probed with the antisense strands for both osteopontin and cathepsin K (Figs. 6A and 6B). The strong hybridization signals were also detected over multinucleated osteoclasts, and a population of mononuclear cells in sections of osteoclastoma tissue (Figs. 6D and 6E). In contrast, no signal was detected over the 87MEM1-positive cells, or osteoclastoma tissue sections, that had been incubated with the sense strands of either probe (Figs. 6C and 6F).

Induction of resorption

The definitive characteristic of an osteoclast, which makes it unique from any other cell type, is its ability to resorb bone or dentine. The 87MEM1-positive cells were cultured on dentine in medium supplemented with $1,25(OH)_2D_3$ (10^{-8} M) and 70% osteoclastoma-conditioned medium. Although these cells did express high TRAP activity, they did not resorb pits in the dentine. Subsequently, the 87MEM1-positive cells were cocultured

FIG. 6. (Opposite) Osteopontin and cathepsin K mRNA by in situ hybridization. (A) A strong hybridization signal was detected on the 87MEM1-positive cells probed with the osteopontin antisense strand (arrowheads). Autoradiography/methylene blue counterstain. Bar = 10 μm. (B) In addition, the signal obtained from the cathepsin K antisense probe was also strong and selective for the 87MEM1-positive cells (arrowheads). Autoradiography/methylene blue counterstain. Bar = 10 μm. (C) No nonspecific signal was observed in the 87MEM1-positive cells probed with the Cathepsin K sense strand negative control. Bar = 10 μm. (D) Osteoclasts (arrowheads) and mononuclear cells demonstrated a strong hybridization signal in cryostat sections of the native tumor screened with the cathepsin K antisense probe. Autoradiography/methylene blue counterstain. Bar = 100 μm. (E) A higher magnification clearly shows the presence of a cathepsin K–positive mononuclear cell population in the native tumor. Autoradiography/methylene blue counterstain. Bar = 10 μm. (F) No nonspecific signal was observed against osteoclasts (arrowheads) or mononuclear cells on sections of the native tumor incubated with the sense strand of cathepsin K. Autoradiography/methylene blue counterstain. Bar = 50 μm.

FIG. 7. (Opposite) Resorption of pits in whale dentine slices. (A) Extensive resorption could be detected on the surface of the dentine slice cultured with the 87MEM1-positive cells. The extent of resorption varied from small, discrete pits (small arrows) to very large, amorphous excavations (large arrows). Scanning EM. Bar = 100 μm. (B) Apart from the abrasions caused by the saw that cut the slices, no other imperfections, due to resorption, could be detected on the dentine slice that had been cultured with stromal cells alone. Scanning EM. Bar = 100 μm. (C) Stromal cells and osteoclasts cocultured on the dentine slice are shown. The resorbing osteoclasts (arrows) can be observed within and adjacent to resorption lacunae (small arrows). A stromal cell can be seen in the top righthand corner of the photograph (long arrows). Scanning EM. Bar = 25 μm. (D) Size distribution of the resorption pits excavated by the 87MEM1-positive cells. These ranged from 5 to 35 μm and indicates that the majority of the resorption was performed by either mononuclear or small multinucleated osteoclasts. (E) Osteoclastoma-derived osteoclasts were seeded onto dentine slices for 48 h. There was a larger range in pit size than was detected with the 87MEM1-positive cells, and the majority of pits were also larger than those excavated by the 87MEM1-positive cells.

with osteoclastoma-derived stromal cells, under the same conditions described above, and the dentine slices demonstrated the presence of numerous TRAP-positive cells and large areas of resorption after 7 days in culture (Fig. 7A). Stromal cells cultured alone were negative for TRAP activity and were unable to excavate pits in the dentine (Fig. 7B). When the stromal cell layer was partially removed (by agitation in PBS) from the dentine seeded with the cocultured cells and was analyzed by scanning electron microscope (EM), a large population of resorbing cells was detected (Fig. 7C). The resorption pits on the coculture dentine were analyzed by both laser confocal microscopy and scanning EM to determine their diameter and depth. Since both methods gave comparable results, the following data were generated from the laser confocal microscope by analysis of 69 randomly chosen pits that could easily be measured and attributed to individual osteoclasts. These data were compared with a similar number of resorption pits produced by mature multinucleated osteoclasts derived from collagenase digests of human osteoclastoma tissue. The pits excavated by the 87MEM1-positive cells varied in diameter from 7 to 25 μm (mean 14.3 μm), and the average depth of these pits was 2.1 μm (range 1–4.2 μm). The pits excavated by the mature multinucleated osteoclasts varied in diameter from 10.5–63 μm (mean 27.4 μm), and the average depth of these pits was 2.96 μm (range 1.8–6.5 μm). The distribution of the pit sizes is shown in Figs. 7D and 7E for the 87MEM1-positive cells and mature osteoclasts, respectively. In addition to the well-defined excavations in the 87MEM1-positive cell cocultures, there were also extensive areas of resorption that could not be attributed to a definite number of osteoclasts (Fig. 7A). Twelve of these areas were analyzed and varied in diameter from 30.8 to 192.7 μm and had an average depth of 4.1 μm (range 2.2–6.0 μm).

DISCUSSION

In this study, we describe the use of osteoclastoma-derived cell suspensions as a source of human osteoclast precursors. Based on previous observations that mononuclear cells in this tissue express the vitronectin receptor, a monoclonal antibody to its β_3 chain was used to localize and ultimately isolate this putative precursor population.[8] Subsequent analysis of this isolated population of cells demonstrated that it not only expresses a number of osteoclast phenotypic characteristics, but significantly, can also be induced to resorb pits in dentine, the ultimate function that defines an osteoclast.

It was demonstrated that the 87MEM1-positive cells express TRAP activity that appeared to be up-regulated when the cells were cultured on dentine slices. In contrast, isolated peripheral blood monocytes did not express this TRAP activity but, unlike the 87MEM1-positive cells, did express high levels of NSE (a marker for cells of the monocyte lineage). There have been reports that TRAP enzyme activity can be detected in cultured monocytes after short incubation times in culture.[20] This discrepancy in detecting activity may, however, be attributed to the long reaction times used by these workers. The short reaction time (4 minutes) employed in this study provides selectivity between the two cell types.

Calcitonin receptors, which, in the bone marrow environment, are believed to be found exclusively on osteoclasts,[21–23] were also detected on the 87MEM1-positive cells. Indeed, this selective marker has been used to distinguish osteoclasts from the morphologically similar macrophage polykaryon.[24,25] In recent studies, it has been demonstrated that calcitonin mRNA and protein is expressed in murine osteoclasts prior to multinucleation.[26]

By in situ hybridization, 87MEM1-positive cells demonstrated abundant expression of cathepsin K, a novel cysteine proteinase that has been described recently by four groups,[14–17] who demonstrated its selectivity for osteoclasts. The enzyme has endoprotease activity against fibrinogen at acid pH, and it is proposed that it may play a vital role in matrix degradation.[16] A positive signal was also observed over osteoclasts and a population of mononuclear cells in both cryostat sections of the native osteoclastoma tissue and in adult osteophytic bone. In addition to the cathepsin K mRNA expression, we have also recently demonstrated selective cathepsin K protein expression in osteoclasts and a population of mononuclear cells in cryostat sections of osteoclastoma and osteophyte.[27] The cathepsin K positive mononuclear cells in the osteophyte were often associated with osteoclasts while the remainder of the bone marrow in these sections was negative.

The osteoclastic phenotype of the 87MEM1-positive cells is further supported by their reactivity with human osteoclast-selective monoclonal antibodies, 87MEM1 and C35, and their concomitant lack of reactivity with antibodies that recognize cells of other mature hematopoietic cell types. The 87MEM1 antibody, reactive with the β_3 chain of the vitronectin receptor,[5] was used to isolate the precursor population, since it has been widely reported that osteoclasts are a major expressor of this receptor in hematopoietic tissues. We have been unable to detect binding of this antibody to monocytes or macrophages. Similarly, C35 reacts selectively with an unknown cytoplasmic antigen present in osteoclastoma and human bone tissue–derived osteoclasts, but does not react with peripheral blood monocytes or macrophages from a number of tissue sites.[8] Recent data from our laboratory, derived from in situ hybridization and histochemical studies on human osteophytic bone, suggest that mononuclear cells in resorption lacunae express TRAP activity and osteopontin mRNA.[28] The evidence suggests that these cells, which appear to differentiate from TRAP negative/NSE positive mononuclear cells, ultimately fuse to form multinucleated osteoclasts.

The data from the resorption experiments support the contention that osteoclast differentiation cannot occur in the absence of a stromal cell layer.[29–31] The variability in the size of the resorption lacunae, compared with those excavated by multinucleated osteoclasts in a routine human resorption assay, suggest that they were excavated by mononuclear as well as by multinucleated osteoclasts. This observation is not without precedent, since resorbing mononuclear osteoclasts have been detected in sections of human osteophytic bone.[28]

Human osteoclast-like cell lines have been previously reported in the literature and consist of those derived from long-term marrow cultures[32] or from leukemic cell lines, such as the phorbol ester–induced osteoclast-like cell line, FLG 29.1[1] and HL-60 cells.[2] The long-term marrow cultures have the disadvantage of consisting of heterogeneous populations of cells, which can confuse the issue of effector cell identification when testing regulatory agents. Also, we were unable to observe reactivity of osteoclast-selective monoclonal antibodies with the multinucleated cells formed in these cultures.[8] The leukemic cell–derived osteoclast-like cell lines express a number of osteoclast phenotypic characteristics, including multinucleation, TRAP activity, and the presence of calcitonin receptors. However, in the case of the FLG29.1 cells, they do not express a ruffled border and consequently do not possess the ability to resorb pits in dentine slices.[1] In addition, in our experiments, they do not show C35 reactivity.

HL-60 cells can be induced to form osteoclast-like cells under the appropriate conditions.[2] This process requires the formation of colonies in methylcellulose, followed by sequential culture in $1,25(OH)_2D_3$-supplemented medium and then tumor-conditioned medium derived from a squamous cell carcinoma cell line. The result of these complicated manipulations is a population of cells that express a number of osteoclastic characteristics but are unable to demonstrate efficient resorption on whale dentine (10 pits/10^5 cells). In contrast, innumerable osteoclastic pits were excavated by 2×10^5 cells described in this study.

In conclusion, the results of this study demonstrate that osteoclastoma tissue provides an excellent source of osteoclast precursors. Using an antibody to the β_3 chain of the vitronectin receptor, in conjunction with flow cytometry, we are able to reproducibly purify the precursor population. The commitment of these cells to the osteoclast lineage is defined by their ability to resorb pits in dentine slices, providing an excellent model for studying human osteoclast differentiation.

ACKNOWLEDGMENTS

We thank Dr. Fred Drake, Dr. Mark Nuttall, and Mr. David Rieman for critically reviewing this manuscript and for their helpful comments.

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Received in original form March 6, 1996; in revised form May 14, 1996; accepted July 1, 1996.